APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1996, p. 3521–3526 Vol. 62, No. 9
0099-2240/96/$04.00+0

A Quantitative Method To Evaluate Neutralizer Toxicity against *Acanthamoeba castellanii*

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Received 20 February 1996/Accepted 20 June 1996

A standard methodology for quantitatively evaluating neutralizer toxicity against *Acanthamoeba castellanii* does not exist. The objective of this study was to provide a quantitative method for evaluating neutralizer toxicity against *A. castellanii*. Two methods were evaluated. A quantitative microtiter method for enumerating *A. castellanii* was evaluated by a 50% lethal dose endpoint method. The microtiter method was compared with the hemacytometer count method. A method for determining the toxicity of neutralizers for antimicrobial agents to *A. castellanii* was also evaluated. The toxicity to *A. castellanii* of Dey-Engley neutralizing broth was compared with Page's saline. The microtiter viable cell counts were lower than predicted by the hemacytometer counts. However, the microtiter method gives more reliable counts of viable cells. Dey-Engley neutralizing medium was not toxic to *A. castellanii*. The method presented gives consistent, reliable results and is simple compared with previous methods.

Acanthamoeba keratitis is a severe and potentially sight-threatening ocular infection associated with contact lens wear (2, 17, 18, 27) and is more prevalent with daily-wear soft contact lenses (19). The *Acanthamoeba* organism has been isolated from diverse environmental sources, particularly water sources (16) and can exist in two forms: the motile trophozoite and the double-walled cyst (3, 4). In the encysted state, they are protected from unfavorable environmental conditions and are resistant to killing by freezing (6), desiccation (23), and numerous antimicrobial agents (20). Since the accelerated incidence of contact lens wear-related *Acanthamoeba* keratitis in the mid 1980s (27), several methods for testing contact lens disinfection systems against *Acanthamoeba* organisms have appeared in the literature. The lack of a standard methodology for testing disinfectants against *Acanthamoeba* organisms results in confusion and inconsistencies regarding the effectiveness of the disinfection systems (14, 15). A variety of methods have been utilized to evaluate contact lens disinfection systems against *Acanthamoeba* organisms and to neutralize the active ingredients in the systems. These methods include studies of the presence or absence of motile trophozoites after disinfectant exposure and neutralization with Norton's neutralizer containing catalase and sodium thiosulfate (12); hemacytometer counts of cells after 24 h of disinfectant exposure (5); exposure times followed by washing of cells from challenged solutions with phosphate-buffered saline (30) or neutralizing Page's saline containing 0.7% lecithin and 0.5% Tween 80 (24), with subsequent plating onto bacterial lawns; and standard plaque assays succeeding disinfectant exposure and washing with 0.15% KCl (10) or a nonspecific neutralizing solution (11). Also, stains such as lactophenol cotton blue have been used for rapid detection of cysts in tissue samples. However, trophozoites did not stain as well as cysts (29). It is evident that there is a need for standardized methods for the assessment of contact lens disinfection systems against *Acanthamoeba* organisms.

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In the present study we have developed a quantitative technique for evaluating neutralizer toxicity against *Acanthamoeba castellanii* trophozoites by using microtiter plates, detection of amoeba tracks on non-nutrient agar plates overlaid with a bacterial lawn for viability determination, and the 50% lethal dose endpoint computation developed by Reed and Muench (26) for quantitating the number of survivors. Because of the use of antimicrobial agents in contact lens care products, the toxicity to *Acanthamoeba* organisms of antimicrobial neutralizers was a concern. A modified neutralizer toxicity evaluation found Dey-Engley neutralizing broth to be suitable for *Acanthamoeba* recovery, and therefore, this broth could be used to facilitate the neutralization step needed in testing of contact lens disinfecting solutions. The Dey-Engley neutralizing broth contains many of the neutralizers necessary for testing most of the contact lens disinfection systems available today. Our method combines a technique for viability determination, an accurate quantitation method, and a neutralizing medium for testing disinfectants against *A. castellanii.*

Organism. Axenic cultures of *A. castellanii* (ATCC 30234) were obtained from the American Type Culture Collection (Rockville, Md.). All procedures were performed in a biological safety cabinet, and safety guidelines pertinent to working with *Acanthamoeba* organisms were followed.

Media and reagents. Peptone-yeast extract-glucose medium (PYG) was used to grow the cultures of *A. castellanii* (21). Dey-Engley neutralizing broth (DE broth) (Difco) was used as the neutralizing dilution blanks. DE broth contains sodium thioglycolate, sodium thiosulfate, sodium bisulfite, Polysorbate 80, and soybean lecithin to neutralize the mercurials, halogens, aldehydes, and phenolic and quarternary ammonium compounds, respectively (7, 8). The composition of DE neutralizing broth is presented in Table 1. Page's saline was used as the control dilution blanks (9).

Growth of *A. castellanii*. Stocks of *A. castellanii* cysts maintained at −70°C were gradually thawed and axenically grown by inoculation into a 150-cm^2 tissue culture flask containing 100 ml of PYG broth for approximately 7 to 10 days to obtain motile trophozoites. The organisms were then axenically subcultured in PYG broth for 7 to 10 days at 30 to 35°C. The tissue culture flasks were scraped with a sterile cell scraper to dis-

TABLE 1. Dey-Engley neutralizing broth[a]

Neutralizer	Composition (%) in broth	Chemical to be neutralized
Sodium thioglycolate	0.1	Mercurials
Sodium thiosulfate	0.6	Halogens
Sodium bisulfite	0.25	Aldehydes
Polysorbate 80	0.5	Phenolic compounds
Lecithin (soybean)	0.7	Quaternary ammonium compounds

[a] Data summarized from Engley and Dey (8). Dey-Engley neutralizing broth (Difco) also contains tryptone (0.5%), yeast extract (0.25%), glucose (1.0%), and bromcresol purple (0.002%).

lodge adherent organisms and gently rocked back and forth to resuspend the scraped cells. Suspensions were prepared by centrifugation of the PYG broth suspension at 1,300 × *g* for 20 min at room temperature in a Sorvall centrifuge (model RT6000B; Sorvall Instruments) and decanting the PYG broth to leave a pellet of amoeba. The pellet was resuspended in Page's saline.

Hemacytometer counts. The amoeba suspension was diluted 1:10 to facilitate counting with a hemacytometer (Levy chamber; Hausser Scientific). The hemacytometer count was performed to get a general idea of the initial concentration of amoeba in the inoculum on day 0, since the bacterial overlay plate counts would not be determined until day 14. The hemacytometer chamber was loaded with the amoeba suspension and counted on a microscope (model FX.35A; Nikon) at a magnification of ×100. Staining was not performed. The suspensions contained 1.0×10^5 to 1.0×10^7 amoeba per ml after adjusting for the dilution factor. A hemacytometer count of 1.0×10^6 amoeba per ml correlates to a count of 1.0×10^4 track-forming units (TFU) per ml from the Page's saline dilution or DE broth dilution, since a 1:100 dilution in the respective diluents was made. The hemacytometer count included viable and nonviable amoeba.

Preparation of bacterial lawns. This procedure uses nonnutrient agar overlaid with an *Escherichia coli* (ATCC 8739) lawn to detect viable organisms. The *E. coli* cells were inoculated into a sterile flask containing 100 ml of Trypticase soy broth (TSB) 48 h prior to testing. After 24 h of incubation at 30 to 35°C, the *E. coli* cells were centrifuged at 1,300 × *g* for 20 min at room temperature. Trypticase soy broth was decanted, and the bacteria were resuspended in Page's saline. With a sterile pipette, each of the wells of the flat-bottom six-well tissue culture plates containing 4 ml of solidified non-nutrient agar in each well received 0.2 ml of the bacterial suspension. The plates were gently tilted back and forth in order to evenly distribute the bacterial suspension. The plates were left unstacked at room temperature overnight to allow Page's saline to evaporate, leaving a smooth, dense lawn of bacteria.

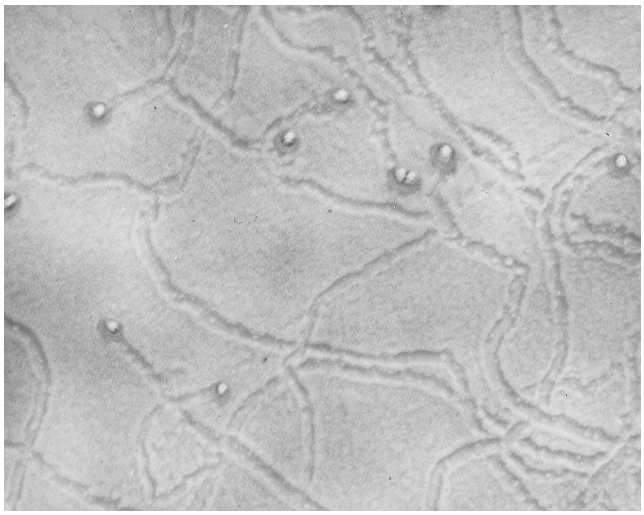

FIG. 1. *Acanthamoeba* track formations magnified ×100.

Procedure for the assessment of toxicity to *A. castellanii* of neutralizing medium. An *A. castellanii* suspension was inoculated into tubes containing 10 ml of Page's saline with 0.1 ml of the inoculum (1:100 dilution) and further diluted by removing 0.1 ml of the solution and immediately diluting in tubes containing 0.9 ml of DE broth (1:10 dilution). Similarly, an inoculum control was prepared by inoculating 10 ml of Page's saline and diluting in Page's saline (1:10 dilution). This also served as a positive control. The tubes containing 10 ml of

TABLE 2. Example of raw data and computation of log survivors

Dilution	Reading[a] on day 14 in well:				Computation[b]					
					Positive	Negative	Total		Ratio	%
	1	2	3	4			Positive	Negative		
10^{-1}	+	+	+	+	4	0	11	0	11/11	100
10^{-2}	+	+	+	+	4	0	7	0	7/7	100
10^{-3}	+	+	−	−	2	2	3	2	3/5	60
10^{-4}	+	−	−	−	1	3	1	5	1/6	17
10^{-5}	−	−	−	−	0	4	0	9	0/9	0

[a] Final observations were made on day 14. Symbols: +, tracks were present in the well; −, tracks were absent.
[b] Calculated according to Reed and Muench (26) as shown below:

(i) Proportionate distance = (percent survival at dilution next above 50%) − 50%/(percent survival at dilution next above 50%) − (percent survival at dilution next below 50%)

(ii) log survivors = proportionate distance + dilution factor next above 50%

Example:

(i) $$\frac{(60 - 50)}{(60 - 17)} = \frac{10}{43} = 0.23$$

(ii) $10^{0.23} + 10^{3.0} = 10^{3.23} = \log 3.23$ or 1.7×10^3 TFU/ml

TABLE 3. Comparison of Page's saline and DE dilution counts of *A. castellanii*[a]

Test no.	Log_{10} count		
	Hemacytometer[b]	Page's dilution[c]	DE dilution[d]
1	4.0	3.3	3.2
2	4.3	2.5	3.5
3	4.3	3.8	3.5
4	4.9	4.8	3.7
5	5.1	4.3	4.5
6	5.1	4.5	4.5
7	5.3	4.3	4.3
8	5.1	4.8	4.7
9	5.1	4.5	3.7
Mean ± SD	4.8 ± 0.5 (A)[e]	4.1 ± 0.8 (B)	4.0 ± 0.5 (B)

[a] Tests performed with trophozoites. All counts (excluding the hemacytometer counts) were done by diluting the samples and plating in quadruplicate on non-nutrient agar with an *E. coli* overlay. The Reed-and-Muench computation (26) was used to calculate the final counts.

[b] Theoretical hemacytometer count after a 1:100 dilution. Viable and nonviable amoeba were detected.

[c] The Page's saline dilution consisted of 0.1 ml of the initial count from the hemacytometer inoculated into 10 ml of Page's saline and diluted in Page's saline.

[d] The DE broth dilution consisted of 0.1 ml of the initial count from the hemacytometer inoculated into 10 ml of Page's saline and diluted in Dey-Engley neutralizing broth.

[e] Analysis of variance with least significant difference *t* test ($F = 5.05$, $P = 0.0148$, degrees of freedom = 2,24). Means with the same letter in parentheses are not significantly different.

Page's saline were thoroughly mixed for 30 s and the dilution blanks were mixed for 10 s with a vortex mixer. Each dilution was plated by removing four 0.1-ml samples and placing them in four separate flat-bottom tissue culture wells with an *E. coli* lawn. All tissue culture plates were taped securely around the edges within 24 h after the test to prevent drying out. The plates were incubated at 30 to 35°C for 14 days.

Procedure for determination and quantitation of survivors. The plates were inspected on days 2, 7, and 14 with an inverted microscope (DIAPHOT-TMD; Nikon) at a magnification of ×100 for the presence of tracks. As the amoeba migrated over the agar surface engulfing the *E. coli* cells that formed the bacterial lawn, the agar underneath became visible, resulting in the formation of tracks. TFU refers to the amoeba that formed the track. TFU per milliliter represented the number of viable amoeba per milliliter from the tube that contained 10 ml of Page's saline which was plated on bacterial overlay plates and correlated with the number of amoeba per milliliter from the hemacytometer count; however, the hemacytometer count included nonviable amoeba and viable amoeba and therefore was consistently higher. Figure 1 shows *A. castellanii* track formations on an *E. coli* lawn magnified 100×. Table 2 shows an example of how raw data was recorded. Table 2 also shows how the Reed-and-Muench computation was applied to the raw data to quantitate surviving organisms. If initial and interval inspections showed a well to be negative, it was not recorded as negative unless it was still negative on day 14. Positive wells were recorded as soon as detected.

Observations. The hemacytometer counts were derived directly from the hemacytometer and included viable and nonviable cells because viability is a distinction that cannot be determined with the microscope. The numbers of viable or-

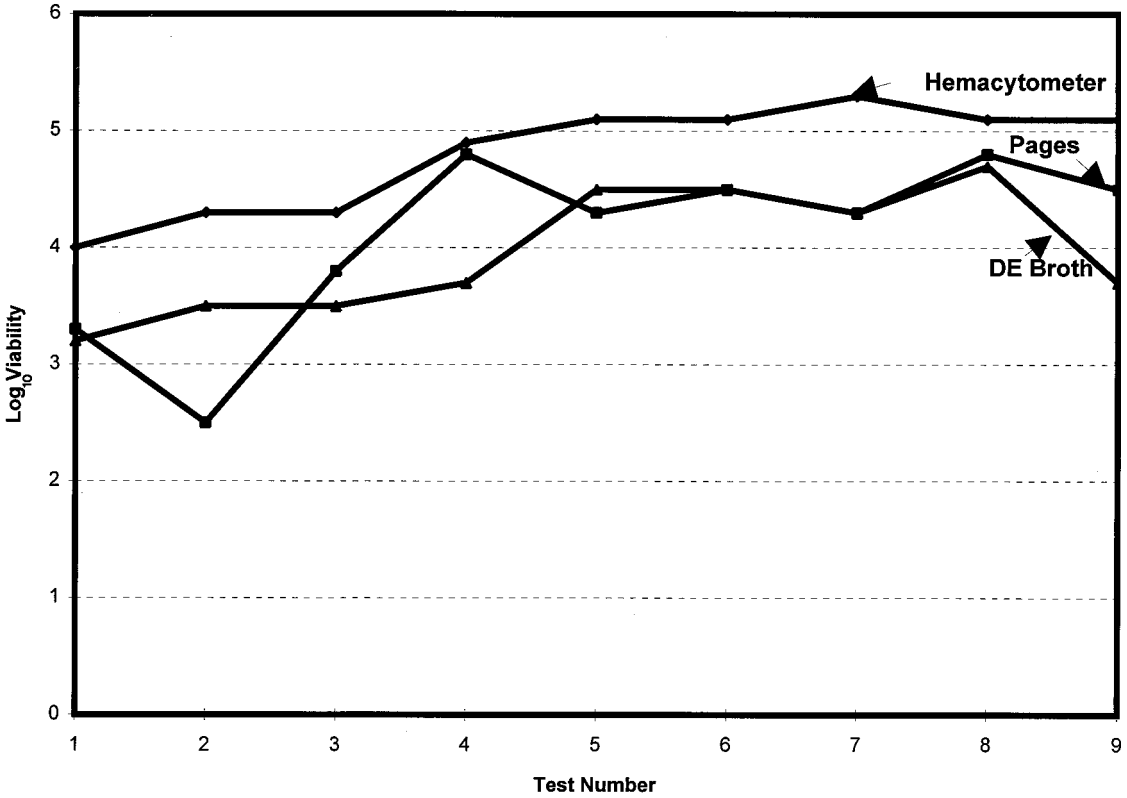

FIG. 2. Comparison of recovery methods for *A. castellanii.*

TABLE 4. Comparison of methods used for *Acanthamoeba* testing

Study	Quantitation	Viability detection
Larkin et al. (12)	Positive and negative results only	Bacterial overlay plates
Connor et al. (5)	Hemacytometer	Organisms left for 1 week in tryptose phosphate broth and recounted
Zanetti et al. (30)	Percentage of viable amoeba determined by degree of growth compared with controls	Bacterial overlay plates
Penley et al. (24)	Degree of turbidity observed for lenses; positive and negative results for solutions	Bacterial overlay plates
Hugo et al. (10)	Standard plaque counting assay	Agar sandwich technique
Kilvington et al. (11)	Plaque counting	Bacterial overlay plates
Ludwig et al. (13)	Positive and negative results only	Bacterial overlay plates
Nauheim et al. (22)	Hemacytometer	Bacterial overlay plates
Perrine et al. (25)	Most probable number	Bacterial overlay plates
This study (quantitative method for evaluating neutralizer toxicity)	Reed-and-Muench computation	Bacterial overlay plates

ganisms from Page's saline dilution and DE broth dilution were determined with microtiter plates. The 50% lethal dose endpoint method developed by Reed and Muench (26) was used to determine counts from the Page's saline dilution and DE broth dilution microtiter plates.

A comparison of the hemacytometer counts, the Page's saline dilution counts, and the DE broth dilution counts for nine separate tests are summarized in Table 3. The average $\log_{10}$ count on the hemacytometer was 4.8 ± 0.5. The hemacytometer count was consistently higher than the Page's saline dilution count and the DE broth dilution count, because the hemacytometer count included dead organisms as well as viable organisms. Once the organisms were plated on non-nutrient agar with an *E. coli* lawn, only viable organisms were detected. The Page's saline dilution count and the DE broth dilution count were within 0 to 1.0 $\log_{10}$ of one another, as shown in Fig. 2. Most values ($\log_{10}$ counts) were within 0.5 $\log_{10}$ with the average $\log_{10}$ count for the Page's saline dilution count being 4.1 ± 0.8 and the DE broth dilution $\log_{10}$ count being 4.0 ± 0.5. The means for the Page's saline dilution and the DE broth dilution were not significantly different.

The amoeba tracks were easily seen with an inverted microscope. Results were recorded for each tissue culture well, allowing titers to be determined by the Reed-and-Muench computation. The data showed that DE broth was suitable for recovering *A. castellanii.*

Discussion. Standardized methods for testing contact lens disinfection systems against *Acanthamoeba* organisms are needed in order to effectively evaluate the systems. An accurate quantitation technique that also detects viability, as well as an antimicrobial neutralization method that allows for successful recovery of the organism, is necessary. Table 4 shows the advantages and disadvantages of present *Acanthamoeba* testing methods.

The active ingredients in contact lens disinfecting solutions can be neutralized either by dilution or by use of specific reagents that inhibit their activity. Previous studies have used a variety of methods to halt the antimicrobial action against the organism. Single and multiple centrifugations and washings of samples to remove the antimicrobial agent have been used (13, 22, 30), adding the risk of possibly weakening the organism (1). Another study used dilution of the antimicrobial agent to fa-

TABLE 4—*Continued*

Neutralization	Advantage(s)	Disadvantage(s)
Dilution using Norton's neutralizer modified with catalase and sodium thiosulfate	Viability determined; disinfectant neutralization accomplished	Quantitation not determined; microscopic examination
Not performed	Hemacytometer is easy to use and provides rapid enumeration of amoeba	Hemacytometer count will not reveal accurate count of viable amoeba; organisms had to be counted a second time after 1 week; disinfectant neutralization not performed—possible stasis
Amoeba washed with phosphate-buffered saline and resuspended in PYG broth	Viability is determined	Quantitation not accomplished; microscopic examination; disinfectant neutralization not performed—possible stasis
Amoeba washed and resuspended in Page's saline containing 0.7% lecithin and 0.5% Tween 80	Viability determined; neutralizer used	Quantitation not accomplished; limited neutralizer efficacy
Amoeba washed once and resuspended in 0.15% KCl	Quantitation and viability determination accomplished	Plaques can be difficult to read if bacterial overlay is too dense or sparse; air bubble interference; disinfectant neutralization not performed—possible stasis
Amoeba suspended in nonspecific neutralizer and washed twice with Page's saline	Quantitation and viability determination accomplished	Multiple centrifugations might weaken the organism; plaques can be difficult to read if bacterial overlay is too dense or sparse; air bubble interference; limited neutralizer efficacy
Amoeba washed three times and resuspended in Page's saline	Viability determined	Quantitation not accomplished; multiple centrifugations might weaken the organism; microscopic examination; disinfectant neutralization not performed—possible stasis
Amoeba washed twice and resuspended in nutrient broth	Viability determined; hemacytometer is easy to use and gives rapid enumeration of amoeba	Hemacytometer count will include viable and nonviable amoeba; multiple centrifugations might weaken the organism; microscopic examination; disinfectant neutralization not performed—possible stasis
Multiple washings and dilution with phosphate-buffered saline	Quantitation and viability determination accomplished	Multiple centrifugations might weaken the organism; microscopic examination
DE broth	Quantitation, viability determination, and neutralization accomplished; DE broth already contains most of the necessary neutralizers, making preparation of media simple	Microscopic examination

cilitate neutralization (25). Separate studies have employed the use of neutralizing media, such as Page's saline with lecithin and Tween 80, as well as neutralizing media to which catalase and sodium thiosulfate was added. These media are sufficient for neutralizing antimicrobial agents. However, the DE broth we used in our study already contains several neutralizers necessary for testing most of the contact lens disinfection systems available today, and therefore preparation of media is comparatively simple. DE broth contains sodium thioglycolate, sodium thiosulfate, sodium bisulfite, Polysorbate 80, and lecithin and may be considered a "universal neutralizing medium."

The specific reagents used for neutralization of antimicrobial agents may be toxic to the test organism, and therefore, it is important to test the growth promotion (neutralizer toxicity) qualities of the neutralizing medium. DE broth was formulated to inhibit the activity of a wide range of disinfectants while allowing bacterial growth. The neutralizer efficacy of DE broth for bacteria has been demonstrated in previous studies (7, 28). Neutralizer toxicity is determined by comparing growth of the organism in the neutralizing medium (without the disinfectant) with growth of the organism in a medium known to be nontoxic to the organism. DE broth was utilized as the neutralizing medium, and Page's saline was used as a medium nontoxic to *Acanthamoeba* organisms. Here we show that DE broth can be used to facilitate the neutralization step needed for testing contact lens disinfection systems against *A. castellanii*, because dilution of challenged test disinfectants in DE broth can halt the antimicrobial action against the organism and allow the surviving organisms to replicate and grow.

Historically, quantitation of *Acanthamoeba* organisms has been performed with hemacytometer counts; however, the viability of organisms is not determined. There have been studies that depicted only the presence or absence of viable organisms by use of bacterial overlays, but enumeration was not accomplished. Other studies utilized a standard plaque counting assay for enumerating survivors. The plaque counting method allowed for viability detection but could give results that are difficult to interpret if the bacterial concentration on overlay plates is too sparse, causing plaques to be too small or difficult to distinguish from air bubbles in the medium. Also, if the bacterial overlays are too concentrated, it is difficult to distinguish the plaques from the dense background (10). Our tech-

nique was flexible in regard to the consistency of bacterial overlays, because the amoeba formed easily detectable tracks with a unique appearance in the bacterial lawns and therefore facilitated rapid determination of the presence or absence of viable *Acanthamoeba* organisms. In the present study, titers were determined by the Reed-and-Muench computation based on assays performed in quadruplicate tissue culture wells. This method produces reliable results that can be used to calculate log reductions and D values (the $\log_{10}$ number of surviving organisms versus time) for contact lens disinfection systems against *Acanthamoeba* organisms.

Our method exemplifies a standard method for testing *Acanthamoeba* organisms because we combine a technique for viability determination, an accurate quantitation method originated by Reed and Muench, and Dey-Engley neutralizing medium for testing contact lens disinfection systems against *A. castellanii* trophozoites.

We thank William M. Bell for technical advice and suggestions relating to this procedure.

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